Modulation of airway epithelial cell function by vitamin D in COPD

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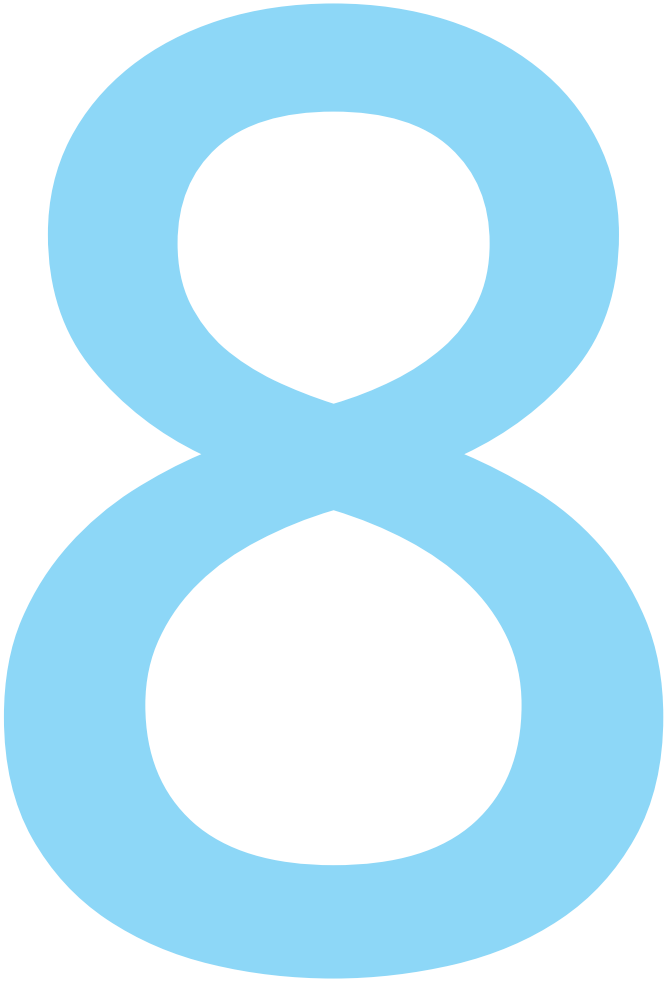
CHAPTER



Summary and General Discussion

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Introduction

In this thesis, a collection of *in vitro* studies is presented that describe the role of vitamin D (biologically inactive 25-hydroxy-vitamin D [25(OH)D] and active 1,25-dihydroxy-vitamin D [1,25$(OH)_2$D]) and inflammatory mediators on respiratory host defense responses and exacerbations, specifically focused on chronic obstructive pulmonary disease (COPD). These *in vitro* studies are followed by a description of a clinical randomized controlled trail [RCT], that aims to investigate in COPD patients whether vitamin D supplementation would indeed be helpful in preventing exacerbations. This thesis is concluded by a review that discusses the latest studies on the effects of disease-associated factors on mucosal vitamin D metabolism and signaling in chronic airway diseases.

The main aim of this thesis is to examine if vitamin D supplementation could be one of the possible strategies to restore the affected host defense responses and could therefore protect against exacerbations in chronic inflammatory lung diseases such as COPD. Before examining the protective role of 1,25$(OH)_2$D in respiratory host defense responses, we first aimed to investigate why respiratory host defense responses are impaired in COPD, by investigating the role of cigarette smoke (CS) and airway epithelial remodeling herein. Furthermore, the presence of disease-associated factors such as inflammation might affect 1,25$(OH)_2$D mediated host defense responses in the lung. We therefore aimed to study the effects of inflammation on the protective role of 1,25$(OH)_2$D in respiratory host defense responses. The aim here was to obtain a better understanding of 1,25$(OH)_2$D signaling and its possible impairment in the lungs of COPD patients, in order to contribute to the discussion on whether (and which) new strategies are needed to enhance local levels of 1,25$(OH)_2$D and protection against exacerbations in COPD.

Overview of the Main Findings in this Thesis

Bacterial- and viral infections are important triggers of COPD exacerbations and the airway epithelium plays a central role in orchestrating immune responses towards invading pathogens (1-4). However, various studies have shown that the physical

and immunological barrier function of the airway epithelium in COPD patients is defective (reviewed in (1, 5)). As a consequence of this airway epithelial dysfunction, the susceptibility towards bacterial- and viral infections is increased and this could further increase exacerbation rate and thereby contribute to disease progression in COPD patients (1, 6, 7). We therefore used *in vitro* culture models of primary airway epithelial cells to study the effects of cigarette smoking and inflammatory mediators on airway epithelial host defense mechanisms, with a specific focus on $1,25(OH)_2D$-mediated epithelial host defense mechanisms. In **Chapter 2,** we first investigated the effects of cigarette smoke (CS) on airway epithelial host defense mechanisms (8). In the studies described in this chapter, we daily exposed airway epithelial cells to whole CS during their differentiation phase to investigate its effects on the expression of constitutively expressed antimicrobial peptides (AMPs) and other host defense mediators such as the polymeric Ig receptor (pIgR). We found that CS exposure reduced the levels of the constitutive expressed host defense mediators SLPI, s/lPLUNC and pIgR and linked this to the inhibitory effects of CS on end-stage epithelial differentiation, i.e. formation of ciliated-, club- and goblet cells. As a result of these inhibitory effects, epithelial antibacterial defenses against *M. catarrhalis* and *K. pneumoniae* were impaired, and also polymeric IgA transport across the airway epithelium was lower after CS exposure. This indicates that CS may have a marked impact on humoral airway epithelial host defense mechanisms through its effect on epithelial remodeling.

In **Chapter 3,** we further explored effects of airway epithelial remodeling on airway epithelial host defense mechanisms, and showed that TGF-β1 caused similar effects as chronic CS exposure on epithelial host defense. Short exposures to TGF-β1 were found to be sufficient to decrease expression levels of the constitutively expressed host defense molecules SLPI, s/lPLUNC and pIgR, as well as numbers of secretory club- and goblet cells (9). We additionally showed that TGF-β1 reduced baseline as well as $1,25(OH)_2D_3$-mediated expression of the AMP hCAP18/LL-37. This inhibitory effect on $1,25(OH)_2D_3$-inducible gene expression could be explained by the observation that TGF-β1 increases degradation of $25(OH)D_3$ and $1,25(OH)_2D_3$ by increasing the expression of the 25(OH)D and $1,25(OH)_2D$-degrading enzyme CYP24A1.

In **Chapter 4**, we further described that long-term exposure of differentiating airway epithelial cells to proinflammatory mediators such as TNF-α, IL-1β and IL-17A, which are elevated in the airways of COPD and refractory asthma patients

(asthma patients, who do not respond to current standard therapy), increased 25(OH)D and 1,25$(OH)_2$D metabolism by enhancing expression of CYP24A1 (10). As a result, the ability of 25$(OH)D_3$ to induce expression of hCAP18/LL-37 and to enhance killing of nontypeable *Haemophilus influenzae* (NTHi) was reduced.

In contrast to **Chapter 3 and 4**, where we showed that 25$(OH)D_3$ and 1,25$(OH)_2D_3$ catabolism was induced and host defense responses in airway epithelial cells were impaired by TGF-β1, TNF-α, IL-1β and IL-17A, in **Chapter 5** we demonstrated that presence of a Th2-inflammatory environment does in fact the opposite (11). We showed that exposure of airway epithelial cells to the Th2 cytokines IL-13 and IL-4 enhanced the expression of CYP27B1, which in turn promoted conversion of the circulating -yet inactive- 25$(OH)D_3$ into the active 1,25$(OH)_2D_3$, thereby inducing expression of hCAP18/LL-37. 1,25$(OH)_2$D treatment was furthermore capable of dampening viral dsRNA-induced inflammatory responses in airway epithelial cells, which was illustrated by a reduction of cytokine- and chemokine release upon Poly(I:C) (a synthetic analog of [viral] dsRNA) stimulation.

To assess whether the afore mentioned protective effects of vitamin D supplementation will indeed reduce exacerbation rate in COPD, a multicenter RCT is designed to study the effects of vitamin D supplementation in a study population that, in contrast to previous RCTs, specifically focusses on vitamin D-deficient patients (serum [25(OH)D] 15 - 50 nmol/L). Study design, protocol and read-outs are described in **Chapter 6** (12).

Finally, in **Chapter 7** we have summarized the current knowledge of the effects of disease-associated factors such as inflammation and cigarette smoke exposure on availability and signaling of vitamin D in the lungs of patients with COPD and other chronic lung diseases. We further aimed to link the possible consequences of an altered 1,25$(OH)_2$D status in the lung to the protective effects of 1,25$(OH)_2$D on mucosal host defense mechanisms, inflammation and exacerbations and explore possible strategies to improve local levels of 1,25$(OH)_2$D.
To answer the question whether vitamin D supplementation could be one of the possible strategies to restore the affected respiratory host defense responses in COPD, we first needed to know how exactly respiratory host defense responses are affected.

Role of Cigarette Smoke and Airway Epithelial Remodeling on Host Defense Responses in COPD

Physical barrier function

Cigarette smoking is an important risk factor for COPD pathogenesis and already in 1957 histological changes in the bronchial epithelium of smokers were described by Chang (13). He described that the bronchial epithelial cell layer was thicker with more stratification and increased numbers of basal and goblet cells, whereas ciliated cell numbers were reduced or had shorter cilia. Our observations in **Chapter 2** showed a marked reduction of cilia development during airway epithelial differentiation when cells were daily exposed to CS (8), and are therefore in line with those of Chang, and the many reports that followed this original observation (14). Moreover, several other studies demonstrated that cilia motility was also impaired in smokers with COPD (14). Expression and function of the cystic fibrosis transmembrane conductance regulator (CFTR) (which maintains optimal apical surface liquid (ASL)- and mucus hydration, volume and pH), is also affected in smokers and in COPD (15). This combination of goblet cell hyperplasia, aberrant expression and function of CFTR and cilia, collectively impairs mucociliary clearance and contributes to airway obstruction and increased susceptibility towards infections (14). Whereas goblet cell hyperplasia is known to be present in smokers and COPD patients, in **Chapter 2** we observed that CS exposure inhibited the formation of goblet cells during epithelial differentiation at the air-liquid interface (ALI) (8). Interestingly, short-term exposure to TGF-β1 was already sufficient to decrease the number of goblet cells in fully differentiated airway epithelial cell cultures (**Chapter 3)** (9). This might indicate that the effect of CS exposure on the expression of mucins and formation of goblet cells could be mediated in part by TGF-β1, which is known to be increased by CS exposure (16). Also, the level of cigarette smoke exposure and the missing immune component could contribute to a lack of goblet cell hyperplasia.

CS exposure furthermore reduces epithelial barrier integrity by causing degradation of its tight and adherens junctions, favoring trans-epithelial invasion of pathogens (7). The loss of expression of tight and adherens junction proteins such as zona occludens (ZO)-1 and E-cadherin, combined with gain of mesenchymal markers, is a hallmark of epithelial-to-mesenchymal transition (EMT) and is present especially in the small airways of (ex-) smokers with and without COPD (17, 18). The effects of chronic CS exposure on airway epithelial barrier integrity were also assessed in

Chapter 2, where only a minor delay was observed in epithelial barrier development during the first week of differentiation, compared to air-exposed cells (8). This was however not significant and could be explained by the fact that decreases in epithelial barrier function (by measuring trans epithelial electrical resistance [TEER]) by CS are transient and normalized at the time of measurement (18-20 h after the previous CS exposure) (19). Altogether, these studies further support the notion that structural epithelial changes may affect physical barrier function in COPD patients and in smokers. Since studies have shown that $1,25(OH)_2D$ may increase epithelial barrier function (see review **Chapter 7**), it would therefore be of interest to investigate if the loss of epithelial junction proteins or epithelial barrier function could be restored by $1,25(OH)_2D$ treatment *in vitro* in CS-exposed airway epithelial cells or by vitamin D supplementation *in vivo* in smokers or COPD patients.

Cellular remodeling

More recent studies have highlighted the importance of club cells in respiratory host defense. Club cells are decreased in COPD patients and by CS exposure *in vitro,* this decrease could negatively impact epithelial barrier function, neutralization of toxins, immunomodulation and epithelial regeneration (20-23). CS furthermore increases production and activation of TGF-β1 in airway epithelial cells and elevated levels of TGF-β1 expression were found in the airways of COPD patients (17, 24-26), although this was not shown in all studies (27). In **Chapters 2 and 3**, we have indeed confirmed that exposure to both CS and TGF-β1 results in a reduction in levels of markers of club cells (8, 9). This could suggest that TGF-β1 might play an important role in the CS-mediated reduction of club cells and club cell-mediated functions in the airway epithelium of COPD patients and smokers. However, to fully determine the role of TGF-β1 in regulating club cell numbers and function (release of CC-10), further studies are needed. This could be achieved by using *in vitro* chronic CS exposure models of airway epithelial cells that are treated with TGF-β1-specific antibodies or TGF-β1-Smad signaling inhibitors followed by assessment of club cell markers. Furthermore, in **Chapter 2** we showed that both ciliated and goblet cells strongly recovered upon cessation of CS exposure, except for club cells that remained absent after almost 1 week of recovery (8). This is in line with studies showing that club cells are more sensitive to damage by CS and other noxious gases than other airway epithelial cell types (28). Elevated TGF-β1-expression levels are known to persist in airway epithelial cell cultures derived from COPD patients, which may help to explain why club cells remain absent after smoking cessation

(29). Future studies could further explore the potential of selective TGF-β1 inhibition in the reduction of club cells and its effects on host defense mediators to consider this as a future treatment strategy in COPD patients.

Humoral respiratory host defense

In addition to the physical barrier, humoral defenses of the airway epithelium are also affected in COPD, and this is at least in part attributable to CS-mediated changes in epithelial composition (30). The humoral barrier is provided by e.g. host defense mediators such as AMPs, reactive oxygen species (ROS), CFTR (which influences pH and therefore activity of AMPs), type I and III interferons and by production of cytokines, chemokines and lipid mediators (31-33). Many of these mediators are directly affected by cigarette smoking or indirectly by CS-induced inflammation or by epithelial remodeling (17, 18, 30, 34). For example, acute CS exposure directly decreases upregulation of inducible AMPs such as human β-defensin-2 (hBD-2) upon triggering by a bacterial component (34), whereas other AMPs such as hCAP18/LL-37 and RNAse7 are increased upon CS exposure (19, 35). Moreover, expression and function of the CFTR is also negatively affected by CS and this process seems to be mediated by TGF-β1 (30, 36). In **Chapter 3** and **4**, we have demonstrated that both chronic CS and short-term TGF-β1 exposures decreased expression of constitutively expressed host defense mediators by affecting airway epithelial cell composition (8, 9). We additionally showed that in particular host defense mediators that are expressed by secretory luminal epithelial cells are reduced upon exposure to CS and TGF-β1 (8, 9). Recent developments in single cell-RNA sequencing confirm that *BPIFB1* (lPLUNC), *SLPI* and *PIGR* are indeed highly expressed by club cells (23, 37). Our results indicate that specifically end-stage differentiation and expression of luminal cell expressed host defense mediators is impaired by chronic CS exposure and, when taking into account the results of **chapter 3**, new research endeavors should focus on investigating whether this is partly regulated by CS-induced expression of TGF-β1. In addition to its effects on airway epithelial antibacterial activity, SLPI and s/lPLUNC are furthermore known to promote respiratory host defense by dampening immune responses, inhibition of proteases and by maintaining apical surface liquid (ASL) hydration (38, 39). Future studies could use more complex culture models, such as an airway-on-chip model with the possibility to expose the cells to airflow and mechanical forces, is a better representation of the *in vivo* situation (40). This model further allows the use of immune cells in combination with well-differentiated airway epithelial cells and

could establish further consequences of both CS exposure and TGF-β1 on other aspects of respiratory host defense such as on immune responses (40, 41).

Vitamin D-mediated host defense responses

Vitamin D ($1,25(OH)_2D$) displays protective effects on host defense responses in the airways, e.g. by maintenance of the of the mucosal barrier integrity, modulation of stress, damage and immune responses and by the promotion of killing of pathogens (e.g. via the induction of the AMP hCAP18/LL-37) (42-44). The VDR is expressed in all epithelial cell types of the lungs with higher expression levels in alveolar epithelial cells and lower expression levels in club cells, compared to other pulmonary epithelial cell types. CYP27B1 is also expressed in all epithelial cell types and higher expression levels are again present in alveolar cells, but lower expression levels are found in both goblet- and ciliated cells. This indicates that all lung epithelial cells, but especially alveolar cells, should be responsive to 25-hydroxy-vitamin D [25(OH)D], the main circulating, yet inactive form of vitamin D (45). Interestingly, expression of CYP24A1 (that converts both 25(OH)D and $1,25(OH)_2D$ into inactive metabolites) is absent in alveolar epithelial cells, but expressed at low levels in club cells and is highly expressed in basal-, ciliated- and goblet cells (45). This suggests that an increase in basal- and goblet cells, as present in the peripheral airways of COPD patients, might increase expression levels of CYP24A1, which degrades $1,25(OH)_2D$ that mitigates its effects on epithelial host defense. Further studies in COPD patients showed that the expression of VDR mRNA and protein in alveolar tissue was gradually reduced in COPD patients with increasing disease severity (46). It would be of interest to determine if changes in expression of genes related to vitamin D metabolism are the result of destruction of alveolar tissue and remodeling of the airway epithelial cell layer, as seen in COPD patients.

In **Chapter 6** we have demonstrated that a pro-inflammatory environment, created by chronic exposure to proinflammatory cytokines such as TNF-α/IL-1β and IL-17A during the differentiation of airway epithelial cells, increased the expression of the $25(OH)_2D$ and $1,25(OH)_2D$-degrading enzyme CYP24A1 without affecting expression of CYP27B1 and VDR (10). We additionally assessed if the changes in CYP24A1 were the result of an altered epithelial differentiation by assessing the effects of TNF-α/IL-1β on the expression of epithelial cell basal, club-, goblet- and ciliated cell markers, and found that expression of these markers was not affected (10). This indicates that TNF-α/IL-1β-mediated increases of CYP24A1 were not explained by

changes in the composition of the epithelial cell layer. Conversely, in **Chapter 4** we found that the TGF-β1-mediated induction of CYP24A1 occurred in concert with a reduction in both club- and goblet cells (47). Despite the fact that similar effects were observed in submerged cultures of undifferentiated airway epithelial cells, we cannot fully exclude that TGF-β1-induced effects on CYP24A1 expression were the result of an altered composition of the epithelial cell layer. To fully elucidate this, airway epithelial cells can be differentiated in presence of stimuli that for example affect end-stage epithelial differentiation, such as CS. Another way is to differentiate cells in presence of a Notch-signaling inhibitor (DAPT), that skews differentiation towards ciliated cells or in presence of IL-13 to increase goblet cell formation. During these processes expression of VDR, CYP27B1 and CYP24A1, and $1,25(OH)_2D$ responses could be monitored at an early/acute phase (after 1 day) and at an later phase of epithelial differentiation (after 3 weeks). In fact, we have already tried this with CS exposure in a pilot study and found that CS inhibited end-stage airway epithelial differentiation as expected and also increased CYP24A1 expression (unpublished observations). Compared to the effects of TNF-α/IL-1β, IL-17A and TGF-β1, the increase in CYP24A1 expression was much less prominent and this might explain why $1,25(OH)_2D$-mediated expression of hCAP18/LL-37 was not affected by CS. In conclusion, both chronic CS exposure and TGF-β1 cause aberrant epithelial remodeling in the conducting airways, thereby disrupting both physical and humoral airway epithelial host defense functions. This might help to explain why smokers with and without COPD have increased susceptibility towards respiratory infections, and how the local environment may determine vitamin D metabolism and action.

Targeting Epithelial Remodeling in COPD

Dysfunctional basal progenitor cells within the airway epithelium are considered to play a fundamental role in the observed aberrant remodeling of the airway epithelium in COPD patients and in smokers and could therefore be a target to reverse this process in COPD (48). Several studies have identified a subset of keratinocyte 5 (KT5)-positive basal cells as basal progenitor cells, and showed that these are characterized by the ability to self-renew and to differentiate into basal-,

secretory-, and ciliated cells (48). COPD patients and smokers with a reduced lung function have lower numbers of $KT5^+$ clone-forming basal progenitor cells than healthy controls, and those cells display signs of exhaustion (49). One of the possible explanations for the observed exhaustion of the basal progenitor cells might be cellular senescence. Senescence is prevalent in both airway- and alveolar epithelial cells in COPD patients and recent studies showed that induction of airway epithelial basal cell senescence results in reduced basal cell proliferation (50-52). Other studies have shown that basal progenitor cells, derived from smokers and COPD patients, have aberrant Notch, TGF-β1, BMP-4 and/or EGFR signaling that also may additionally explain the dysfunctional properties of these progenitor cells (53-56). In our chronic CS-exposure model in **Chapter 3,** we have indeed observed decreases in expression of the Notch-target genes *HEY1* and *HEY2* (but not others) after chronic CS-exposure. In addition, when cells were differentiated in presence of the Notch-signaling inhibitor DAPT, expression of secretory goblet- and club cell markers as well as luminal constitutive host defense mediators was reduced (8), further supporting a role of these pathways in aberrant airway epithelial differentiation. Intervention strategies to treat dysfunctional basal progenitor cells, e.g. by combined targeting of Notch, TGF-β1, BMP-4 or EGFR-signaling pathways, may have the potential to stop or even reverse disease progression. For example, CS-mediated loss of ciliated cells was counteracted through inhibition of EGFR-signaling by Gefinitib during *in vitro* airway epithelial cell differentiation (54). In addition, inhibition of BMP-4 by DMH-1 was able to (partially) reverse CS extract-mediated induction of squamous cell markers and reduction of both ciliated- and secretory cell markers (55), whereas TGF-β1-inhibition during differentiation at the air-liquid interface was able to reverse signs of EMT that were still present in airway epithelial cells, derived from COPD patients (29). It would be interesting to also study effects of such strategies on key enzymes involved in vitamin D metabolism. In **Chapter 3,** we observed that inhibition of TGF-β1-Smad signaling by using SB431542 was sufficient to reverse TGF-β1-mediated reduction of the $1,25(OH)_2D$-inducible hCAP18/LL-37 and this compound also prevented induction of CYP24A1 (9). Perhaps reducing the inflammatory state of the local environment and reducing exposure to inhaled toxicants by smoking cessation could already reverse some features of EMT and other detrimental effects of TGF-β1. It seems that elevated expression levels of EMT-markers could return back to control levels, when COPD-derived airway epithelial cells were cultured at the air-liquid interface for an extended period of time (29). Combining treatment with inhaled corticosteroids

with vitamin D-supplementation could serve to reduce the inflammatory state of the local environment without affecting respiratory host defense responses, which is a well-known side-effect of steroid use (**Chapter 7**, (57)). In conclusion, new approaches to specifically target dysfunctional basal progenitor cells together with improving the local environment could be considered to counteract aberrant airway epithelial remodeling. These new approaches are needed, since remodeling especially of the small airways is currently considered to be a crucial process that precedes further airway obstruction, destruction of alveolar walls and/or development of lung cancer (18, 58).

Modulating of Vitamin D Metabolism in Chronic Inflammatory Lung Diseases

Local availability and action of $1,25(OH)_2D$ depends on expression of VDR and an equilibrium between the vitamin D metabolic enzymes CYP27B1 and CYP24A1, and this can be affected by several inflammatory mediators and exposure to inhaled toxicants, which is discussed in **Chapter 7**. The presence of these inflammatory mediators in chronic inflammatory lung diseases could therefore interfere with expression of VDR, CYP24A1 and CYP27B1, which as a consequence may affect the local levels of $1,25(OH)_2D$. In **Chapter 5** we observed that exposure to Th2 cytokines enhances expression of CYP27B1, thereby promoting 25(OH)D-metabolism in airway epithelial cells, whereas others have shown this effect after exposure to poly(I:C) (11, 60). We have indeed demonstrated in **Chapter 3, 4** and **5** that cytokines that are known to be elevated in both COPD and (refractory) asthma patients, such as IL-4, IL-13, TGF-β1, IL-1β, TNFα and IL-17A, have the ability to alter the expression of genes related to vitamin D metabolism, such as CYP27B1 and CYP24A1 in airway epithelial cells (9, 10, 61). These *in vitro* data suggest that (chronic) airway inflammation can have a large impact on local levels of $1,25(OH)_2D$ through enhanced conversion into $1,25(OH)_2D$ in presence of a Th2-inflammatory environment or enhanced degradation of both 25(OH)D and $1,25(OH)_2D$ in presence of a TGF-β1, Th1-/Th17-inflammatory environment. Nonetheless, airway inflammation in COPD *in vivo* is far more complex and heterogeneous than we have tested so far *in vitro* using monocultures of airway epithelial cells, or using only

immune cells. For that reason, animal models or preferably more complex cell culture models such as organs-on-chips technology in combination with co-cultures of epithelial cells and immune cells that are derived from COPD patients could be used in future studies to fully understand the impact of airway inflammation on $1,25(OH)_2D$ responses and discrepancies between systemic levels of 25(OH)D and local levels of 25(OH)D and $1,25(OH)_2D$. In addition to the demonstrated effects of viruses and cytokines on expression of genes related to airway epithelial vitamin D metabolism (CYP27B1 and CYP24A1), other studies have shown that exposure to inhaled toxicants such as CS and particulate matter (PM) may alter expression or activity of VDR and CYP27B1 (62, 63). This was in contrast to what was observed in most immune cells, such as monocytes and macrophages. In monocytes and macrophages, the effects of $1,25(OH)_2D$ were even enhanced by exposure to most proinflammatory stimuli, apart from Benzo[a]pyrene (BaP), a polycyclic aromatic hydrocarbon produced by cigarette combustion, which increased the expression of CYP24A1 (64-69).

A recent study in COPD patients that assessed both VDR and CYP24A1 expression in parenchymal lung tissue, observed lower expression of both VDR and CYP24A1 compared to non-COPD controls, indicating that also in the lower airways the effects of $1,25(OH)_2D$ might be impaired (70). Importantly, another recent study that investigated vitamin D metabolism in both serum and in lung tissues of both COPD and asthma patients, found evidence to suggest that the conversion of vitamin D_3 into $25(OH)D_3$ was impaired (71). Furthermore, locally in lung tissue and/or in airway epithelial cells derived from both asthma and COPD patients, expression of 25-hydroxylase (CYP27A1), which converts vitamin D_3 into $25(OH)D_3$, was reduced compared to healthy controls, while the opposite was true for VDR. The authors also showed no differences in CYP27B1 expression between groups, which is not in line with our observations *in vitro* of increases in CYP27B1 in presence of Th2 cytokines in **Chapter 5** (11). Furthermore, expression of CYP24A1 was decreased in asthma patients, whereas in lung tissue from COPD patients there was a trend towards a significant increase (log fold change 0.36; $p = 0.06$), which was more in line with our *in vitro* observations in **Chapters 3** and **4** (9, 10). More importantly, another study showed that expression of epithelial $1,25(OH)_2D$-responsive genes was increased in sputum and nasal brushings from asthma patients, whereas this was decreased in lung tissue from COPD patients (72). This study indicates that especially in COPD patients, local levels of $1,25(OH)_2D$ and signaling are indeed impaired and that in addition to CYP24A1, also the expression

of CYP27A1 might play an important role. Collectively, these studies imply that local inflammatory conditions in most cases negatively affect both local levels and activity of $1,25(OH)_2D$ on the airway epithelium.

To enhance local efficacy of $1,25(OH)_2D$, alternative strategies might be considered, such as inhalation with active $1,25(OH)_2D$ or supplementation with $25(OH)D_3$ instead of vitamin D_3, to circumvent the impaired metabolism of vitamin D_3 into $25(OH)D_3$ which is seen in both asthma and COPD patients (71). Also specific CYP24A1 inhibitors, $1,25(OH)_2D$ analogs that are resistant to degradation by CYP24A1, and other VDR agonists can be used, or a combination treatment of vitamin D with anti-inflammatory or certain anti-fibrotic drugs that target TGF-β1 to prevent increases in CYP24A1 (73, 74). It should however be considered that these compounds may enhance the calcemic side effects or impair the immune system. Altogether, we need to fully establish the impact of local inflammation on local $1,25(OH)_2D$ levels and responses by using advanced *in vitro* systems and to some extent animal models, and test these different strategies that may enhance local levels of $1,25(OH)_2D$.

Role of Vitamin D in Airway Epithelial Host Defense Responses

Exacerbations

Exacerbations are a major burden in COPD patients, accelerate decline in lung function and frequently result into hospital admissions and are often triggered by pollutants or by bacterial- and/or viral infections (2-4, 75, 76). COPD patients generally have lower serum 25(OH)D-levels and this is associated with more exacerbations, suggesting that COPD patients might benefit from vitamin D supplementation (77-79). To date, the effects of vitamin D supplementation in the context of COPD exacerbations has been investigated in only 4 RCTs with mixed results: 2 RCTs showed that vitamin D-supplementation reduces the number of exacerbations (80, 81), whereas 2 other RCTs did not find this effect (82, 83). When these 4 RCTs were combined and summarized by using an individual participant data meta-analysis, vitamin D supplementation was shown to be protective against exacerbations in vitamin D deficient COPD patients (baseline serum 25(OH)D levels < 25 nmol/L) (84). This suggests that exacerbations in this group of COPD patients were mainly driven by vitamin D deficiency. To investigate the effects of vitamin D supplementation in vitamin D deficient COPD patients and to gain more insight into

the mechanisms behind the protective effects of vitamin D, the following study was designed: a multicenter RCT that aims to study the effects of vitamin D-supplementation of 6800 IU vitamin D or placebo orally once a week for 1 year on exacerbation rate, systemic and local inflammation, physical performance and quality of life in a study population of vitamin D-deficient patients only (serum [25(OH)D] 15 - 50 nmol/L) (**Chapter 6**) (12). We hypothesize that vitamin D supplementation will improve respiratory host defense responses and will therefore reduce exacerbations and will further improve quality of life in this particular group of COPD patients.

Antibacterial activity

In this thesis, we have identified mechanisms that may underlie the protective effects of vitamin D supplementation *in vitro* in the context of COPD exacerbations, thereby additionally confirming that the airway epithelium is a major target for the effects of locally synthesized $1,25(OH)_2D$. hCAP18/LL-37 is likely to be the most prominent AMP that is induced by $1,25(OH)_2D$ in several types of mucosal epithelial cells and in immune cells, including monocytes and neutrophils (60, 65, 85, 86). The *in vitro* demonstration of $1,25(OH)_2D$-mediated induction of AMPs suggests that vitamin D supplementation could reduce the susceptibility to (respiratory) infections *in vivo.* This was indeed confirmed by several clinical studies, including a recent meta-analysis that showed a protective effect of vitamin D supplementation against acute respiratory tract infections (ARTI) (87). In **Chapter 4,** we have confirmed this ability of $1,25(OH)_2D$ to promote antibacterial activity in differentiated cultures of airway epithelial cells by demonstrating that both 25(OH)D and $1,25(OH)_2D$ increased antibacterial activity against NTHi, a Gram-negative bacterium which is associated with COPD exacerbations (10, 88). We additionally showed that exposure to TNF-α/IL-1β inhibited 25(OH)D and $1,25(OH)_2D$-induced antibacterial effects as well as expression of hCAP18/LL-37, indicating that the 25(OH)D and $1,25(OH)_2D$-mediated antibacterial effects were most likely mediated through the induction of hCAP18/LL-37 (10). Moreover, also other studies have now demonstrated that $1,25(OH)_2D$ treatment increased antibacterial effects against other bacteria such as *E. coli*, *P. aeruginosa* and *B. bronchiseptica* (89, 90). The specific role of hCAP18/LL-37 in the observed antibacterial effects of $1,25(OH)_2D$ on airway epithelium *in vitro* was recently confirmed *in vivo* by Vargas Buonfiglio *et al.*, who demonstrated that the increased antimicrobial activity against the Gram-positive *S. aureus* in ASL after vitamin D supplementation was dependent on presence of hCAP18/LL-37 (63). In addition to

vitamin D, also other strategies of using AMP-inducers such as essential amino acids, sodium butyrate and other (natural) compounds show great potential in their capacity to reduce bacterial infections, especially when some of these compounds are combined (91-93). For example in human colonic epithelial cells (Caco-2), a combination treatment with natural compounds such as andrographolide and isoliquiritigenin synergistically enhanced the AMP human β-defensin-3 (hBD-3) expression and antimicrobial activity, especially against pathogenic bacteria (91). In conclusion, the ability of $1,25(OH)_2D$ to promote bacterial clearance by increasing expression of hCAP18/LL-37 may help to explain why vitamin D deficiency is associated with chronic airway diseases such as asthma and COPD (77, 94).

Inflammation

We have furthermore demonstrated in **Chapter 5** that $1,25(OH)_2D_3$ dampens epithelial release of proinflammatory cytokines and chemokines such as IP-10, RANTES (with a trend towards a decrease in IL-8 and IL-6) that were triggered by stimulation with poly(I:C), a synthetic analog of double-stranded RNA, which is used to mimic viral infections (11). The release of these proinflammatory cytokines and chemokines may further recruit immune cells such as monocytes, eosinophils and neutrophils that secrete proteases and cytotoxic and proinflammatory mediators, which amplifies inflammation, causes alveolar destruction, airway mucus hypersecretion and airway obstruction (95-98). The dampening effect of $1,25(OH)_2D_3$ on the release of cytokines and chemokines in airway epithelial cells was also shown by other studies using other known triggers for COPD exacerbations such as PM, respiratory syncytial virus (RSV), and human rhinovirus (HRV) (99-101). Altogether, these studies indicate that vitamin D supplementation may reduce the severity of exacerbations in COPD by promoting the expression of hCAP18/LL-37 and by dampening the release of proinflammatory cytokines and chemokines in the airways.

Concluding Remarks

Many drivers of COPD pathogenesis such as chronic exposure to noxious particles and gases (including those present in cigarette smoke, biomass fuels or air

pollution), proteolytic enzymes, cytokines and chemokines that are released by inflammatory cells, are known to destruct the epithelial barrier and cause aberrant remodeling of the airway epithelium and as a consequence affect secretion of host defense molecules (7, 102). Therefore, protection and restoration of the epithelial barrier may be a fruitful strategy to halt further disease progression. Firstly, we should consider targeting pathways that restore basal progenitor cell function. Secondly, the local environment should be improved by micronutrients such as vitamin D that protects the airway epithelium from epithelial injury and remodeling. However, we have demonstrated in the studies presented in this thesis that certain airway inflammatory mediators could possibly interfere with vitamin D metabolism by promoting expression of CYP24A1, thereby reducing local levels of $1,25(OH)_2D$ and accompanying protective antimicrobial and anti-inflammatory actions (Figure 1). Therefore, future studies in COPD and but also in other chronic inflammatory diseases that are related to vitamin D deficiency, should be designed with more focus on increasing local levels of $1,25(OH)_2D$ and expression of genes related to vitamin D metabolism, especially CYP24A1. These new insights may yield possible new strategies to target CYP24A1 that enhance local levels and signaling of $1,25(OH)_2D$ to increase protection against exacerbations in COPD patients.

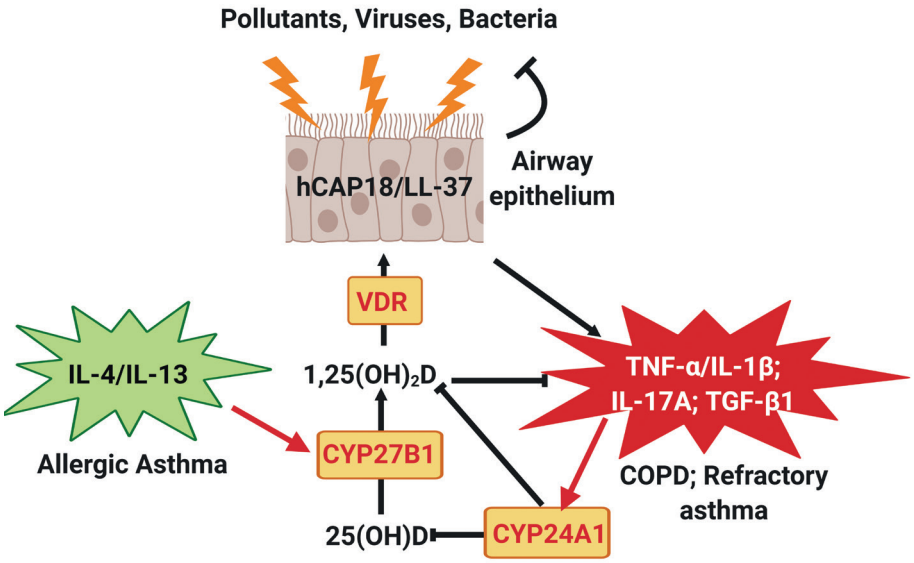

Figure 1. Effects of inflammation on vitamin D-metabolism and epithelial host defense in the airways. Vitamin D receptor, VDR; biologically active vitamin D, $1,25(OH)_2D$; 25-hydroxyvitamin D-1α-hydroxylase, CYP27B1; circulating inactive vitamin D, 25(OH)D; 25-hydroxyvitamin D-24-hydroxylase, CYP24A1

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